OMB No. 0925-0195
Expiration Date 2/28/98

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*: Malaria Vaccine

2. SOLICITATION NO.: PHS 97-2

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*: Birkett, Ashley James

3b. DEGREE(S): B.S. Ph.D.

3c. SOCIAL SECURITY NO.: *Provide on Personal Data Page*

3d. POSITION TITLE: Director of Biochemistry

3e. MAILING ADDRESS *(Street, city, state, zip code)*:
3347 Industrial Court, Suite A
San Diego, CA 92121

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: (619) 793-2661
FAX: (619) 793-2666

BITNET/INTERNET Address:
abirkett@ix.netcom.com

4. HUMAN SUBJECTS
☒ NO ☐ YES
4a. If "Yes," Exemption no. or IRB approval date
☐ Full IRB or ☐ Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS
☐ NO ☒ YES
5a. If "Yes," IACUC approval date: 7-14-94
5b. Animal welfare assurance no.: A-2430-01

6. DATES OF PROJECT PERIOD
From: 7/1/98 *Through:* 12/31/98

7. COSTS REQUESTED
7a. Direct Costs: $ 80,000
7b. Total Costs: $100,000

8. PERFORMANCE SITES *(Organizations and addresses)*
ICC/Synthetic Genetics
3347 Industrial Court, Suite A
San Diego, CA 92121

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Ashley J. Birkett, Ph.D.
ICC/Synthetic Genetics
3347 Industrial Court, Suite A
San Diego, CA 92121

10. ENTITY IDENTIFICATION NUMBER: 1-95-446-7306-A1
Congressional District: 41st

11. SMALL BUSINESS CERTIFICATION
☒ Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified ~~by asterisks (*) or pages~~ on all pages of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☐ YES ☒ NO

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: George B. Thornton
Title: President/CEO
Address: ICC/Synthetic Genetics
3347 Industrial Court, Suite A
San Diego, CA 92121

Telephone: (619) 793-2661
FAX: (619) 793-2666
BITNET/INTERNET Address:
bthorn@ix.netcom.com

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*: A. Birkett
DATE: 12/11/97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*: G. Thornton
DATE: 12/11/97

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

ICC/Synthetic Genetics
3347 Industrial Court, Suite A
San Diego, CA 92121
Telephone: (619) 793-2661

YEAR FIRM FOUNDED	NO. OF EMPLOYEES (include all affiliates)
1994	15

TITLE OF APPLICATION

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
Ashley J. Birkett, Ph.D.	ICC/SG	Principal Investigator

ABSTRACT OF RESEARCH PLAN: State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals and discuss the potential of the research for technological innovation. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. *Therefore, do not include proprietary or confidential information.* DO NOT EXCEED 200 WORDS.

Pre-erythrocytic stage malaria vaccines aim to block hepatocyte entry by sporozoites and/or release of merozoites into the blood stream, thereby circumventing the disease process and rendering the host non-infectious. The primary target of this class of vaccines is the circumsporozoite protein (CS), which is a major constituent of the sporozoite coat when it enters the host. Pre-erythrocytic immunity has been demonstrated by immunization with the CS-NANP repeat epitope, passive transfer of antibodies specific for anti-NANP repeat epitope, and immunization with irradiated sporozoites.

In animal models the immunodominant CS-repeat epitope from either *P.bergeii* or *P.yoelli*, displayed on hepatitis B core particles, successfully protected >90% of animals against infection. Preliminary studies of HBc particles engineered to deliver the CS-repeat from the human parasite *P.falciparum* are promising, but in need of optimization.

The focus of this work is to optimize the immunogenicity of the *P.falciparum* particle to attain the high immunogenicity and absence of genetic restriction observed with *P.bergeii* and *P.yoelli* particles. This will be achieved by optimizing the presentation of the NANP repeat epitope at the surface of HBc and incorporating a universal malaria-specific T cell epitope. Once identified, this vaccine candidate will be the subject of clinical testing during phase II.

Provide key words (8 maximum) to identify the research or technology.

malaria, plasmodium falciparum, circumsporozoite, vaccine

Provide a brief summary of the potential commercial applications of the research.

Malaria is by far the world's most important tropical parasitic disease, and kills more people than any other communicable disease, with the exception of tuberculosis. Malaria is a public health problem in more than 90 countries, inhabited by a total of 2.4 billion people – 40% of the world's population. Mortality due to malaria is estimated to be in the range of 1.5 to 2.7 million deaths each year, accounting for one person every 12 seconds. There are 7 million travelers from the U.S. each year to endemic areas.

Principal Investigator *(Last, first, middle)*: Birkett, Ashley J.

Budget for Phase I—Direct Costs Only

FROM 6/1/98 TO 11/30/98

PERSONNEL *(Applicant organization only)* NAME	Role on Project	Type Appt. *(months)*	% Effort on Project	Institutional Base Salary	DOLLAR AMOUNT REQUESTED *(omit cents)* Salary Requested	Fringe Benefits	TOTALS
Ashley J. Birkett	P.I.	12	100	60,000	30,000	4,500	34,500
Katie Lyons	Tech.	12	100	24,000	12,000	1,800	13,800
Romeo Veniegas	Tech.	12	50	24,000	6,000	900	6,900
SUBTOTALS →					48,000	7,200	55,200

CONSULTANT COSTS

David R. Milich, Ph.D. (30 hrs @ $50/hr)
Ruth S. Nussenzweig, Ph.D. (30 hrs @ $50/hr) — 3,000

EQUIPMENT *(Itemize)*

None — 0

SUPPLIES *(Itemize by category)*

Chemicals and Reagents $7,500
Plasticware and Glassware $2,500 — 10,000

TRAVEL

None — 0

PATIENT CARE COSTS

Inpatient None
Outpatient None — 0

CONTRACTUAL COSTS

0

OTHER EXPENSES *(Itemize by category)*

Animal Purchase/Service (14, 4-Mice Protocols @ $500/per)
Oligonucleotide Synthesis/Purification ($800)
DNA Sequencing ($600)
Electron Microscopy (8 @ $425/per ABI) ($3,400) — 11,800

TOTAL DIRECT COSTS *(Also enter on Face Page, Item 7a)* → $80,000

FIXED FEE REQUESTED — $ 0

OTHER SUPPORT *(see instructions)* [X] NO [ ] YES

Budget Justification

Using continuation pages if necessary, describe the specific functions of the personnel and consultants. Read the instructions and justify costs accordingly.

Personnel/Consultant Costs

Ashley J. Birkett, Ph.D.	Oversee project experimental design, experimentation and data analysis; interact with scientific advisory board members; purification and physical/biochemical analysis of particles; prepare Phase I report and Phase II proposal.
Kate Lynne Lyons, B.S.	Cloning and purification of particles, stability studies, antisera testing.
Romeo Veneigas, B.S.	General laboratory support (reagent preparation, dishwashing, ordering), fermentation.
David R. Milich, Ph.D.	Consult on immunological aspects of projects, including experimental design and data analysis of genetic restriction studies.
Ruth S. Nussenzweig, Ph.D.	Assist in data analysis and selection of the lead candidate. The expertise she has gained from her lifelong studies on malaria and her involvement in previous clinical trials of multiple malaria vaccine candidates will be invaluable to this proposal.

Reagent Costs

Chemicals and Reagents: Cloning reagents (agarose, restriction/modifying enzymes, gel extraction kit, plasmid purification kits); Fermentation media (casein, yeast extract, glucose, ampicillin, agar); Purification reagents (ammonium sulfate, Sepharose CL-4B, HA); Protein Analysis reagents (acrylamide, membranes, SDS, Polaroid film); ELISA reagents (Anti-mouse peroxidase conjugates, TM Blue substrate); Salts (Tris, NaCl, sodium phosphate, etc.)
Plasticware and Glassware: Disposable glass tubes, Eppendorf tubes, pipette tips, serological pipettes, cuvettes, microtitre plates, culture dishes, centrifuge bottles and tubes, chromatography columns, etc.
Animal Services: 14 x 4 Mice Protocols

Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Include laboratory, clinical, animal, computer, and office facilities at the applicant small business concern and any other performance site listed on the FACE PAGE. Identify support services such as secretarial, machine shop, electronics shop, and the extent to which they will be available to the project. Use continuation page(s) if necessary.

ICC/SG's 7500 sq.ft. is located in the Sorrento Valley district of San Diego, proximal to UCSD and The Scripps Research Institute, a popular location for Biotech companies. The facility includes 5000 sq.ft. of wet lab space and 2500 sq.ft. of office space with full-time secretarial, marketing and accounting services available for this project. The company has full internet access in addition to access to scientific literature via the comprehensive bio-medical library at UCSD. The laboratories are fully equipped and engaged in on-going research funded by NIH, NIEHS, product sales from its subsidiary Synthetic Genetics, and private investment. No animal facilities are available on-site; animal studies are routinely contracted out to commercial establishments.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Floor and benchtop centrifuges, microfuges, UV/Vis spectrophotometers, analytic and semi-preparative HPLCs with autosamplers and integrators, kinetic ELISA plate reader, chart recorders, fraction collectors, peristaltic pumps, in-line UV/Vis monitors, computer controlled purification systems (Bio-Rad and PerSeptive BioCad), French press, fume hood, various shakers, waterbaths, PCR thermocyclers, refrigerated chromatography cabinet, -20°C and -70°C degree freezers, agarose and PAGE electrophoresis units, power supplies, darkroom with UV box, gel documentation system, computers, printers, scanner, photocopiers.

A. SPECIFIC AIMS

The focus of this work is to optimize the B cell and T cell immunogenicity of the *P.falciparum* particle such that it has the high immunogenicity and absence of genetic restriction achieved with *P.bergeii* and *P.yoelli*, which were protective to levels of 90-100% in rodents [1, 2]. This will be achieved by optimizing the presentation of the NANP repeat epitope at the surface of the HBc carrier using an improved presentation technique, and incorporating a recently identified universal malaria-specific T cell epitope.

1. Incorporation of the Malaria Specific T cell epitope

HBc is highly effective in enhancing antibody responses to 'carried' epitopes in a manner which shows little or no genetic restriction, therefore ensuring universal antibody priming. This is achieved through a combination of the architecture of the HBc particle, efficient antigen presentation of HBc by B cells and potent T cell recognition of HBc. To ensure the priming of malaria specific T_h cells as well as malaria-specific antibody producing B cells, we intend to incorporate a recently identified universal malaria-specific T cell epitope (Pf Th/Tc) in our vaccine [3, 4].

The importance of including a universal T_h epitope derived from the malaria parasite in a malaria vaccine are several fold. First, the inclusion of an additional T_h epitope will potentially help to increase antibody responses to the NANP epitope in individuals where recognition of the HBc T_h epitope may be limiting. Secondly, the priming of malaria specific T_h cells ensures an animnestic response to *P.falciparum* such that, should a vaccine recipient be exposed to malaria, a more rapid and stronger anti-malaria response will be activated due to previous priming of malaria specific T_h cells. Thirdly, vaccinees living in malaria endemic regions will experience natural 'boosting' every time they are exposed to the parasite because their immune systems have been primed at both the B and T_h cell level. This effect is similar to clinical boosting by re-vaccination – a phenomenon which is particularly attractive for third-world prophylaxis in areas where malaria is endemic.

The malaria-specific T cell site we have selected for inclusion in our vaccine is a universal form of the T cell epitope CS 326-345, recently identified from T cell clones of volunteers who became protected against malaria infection following immunization with irradiated sporozoites [3, 4]. The data presented shows that this epitope appears to bind all human MHC class II molecules and is therefore 'universal' in nature, suggesting that it will prime malaria specific T_h cells in essentially all vaccine recipients. Preliminary reports also indicate that this epitope primes class I restricted T cells. This universal T_h/T_c epitope will be genetically fused to the HBc/NANP particles and tested for its functionality using a variety of *in vitro* and *in vivo* assays.

2. Optimizing Anti-NANP Immunogenicity

The CS protein of *P.falciparum* contains approximately 40 NANP repeats; however, the immunodominant CS epitope is contained within just 3 repeats [5]. To date, our studies have focused on HBc particles bearing 4 repeats at the immunodominant loop region of HBc [1, 2]. We have recently shown that variation of the precise location of the epitope in the immunodominant loop can increase immunogenicity (unpublished observation). Since HBc is a 180 subunit particle, optimum immunogenicity must be determined for this particular presentation system as the conformation of the inserted epitope will vary depending upon the number of repeats presented, which may in turn translate to variable immunogenicity. To address this issue we will investigate the effects of displaying either 3, 6, 9 or 12 NANP repeats at the surface of HBc particles bearing the universal T_h epitope [3]. These particles will be used to immunize mice from multiple genetic backgrounds to investigate immunogenicity and genetic restriction of the anti-NANP immune response.

3. Stability Studies

Two critical concerns of an effective malaria vaccine projected for use in the third world are stability and cost. Cost considerations are discussed later. Initial studies of HBc particles show them to be extremely thermostable, an important consideration given the potential use of this vaccine in developing countries. To address stability of the proposed constructs, accelerated stability studies of the four HBc/NANP particles described in 'Specific Aims 2' will be conducted at 37°C. Particles will be examined by SDS-PAGE to detect proteolysis, electron microscopy to examine particle integrity, ELISA for particle antigenicity, and immunogenicity using B10.S mice. The properties of 'incubated particles' will be critically compared with those stored at –70°C degrees.

Criteria for the Successful Completion of Phase I

The overall aim of the phase I funding period is to optimize the stability and immunogenicity of the NANP/Universal T cell epitope-containing particle. The criteria for the identification and subsequent clinical development (SBIR Phase II) of a candidate particle will be the following:

1. Immunogenicity levels in mice comparable to those achieved in the *P.bergeii* rodent model across multiple genetic backgrounds.

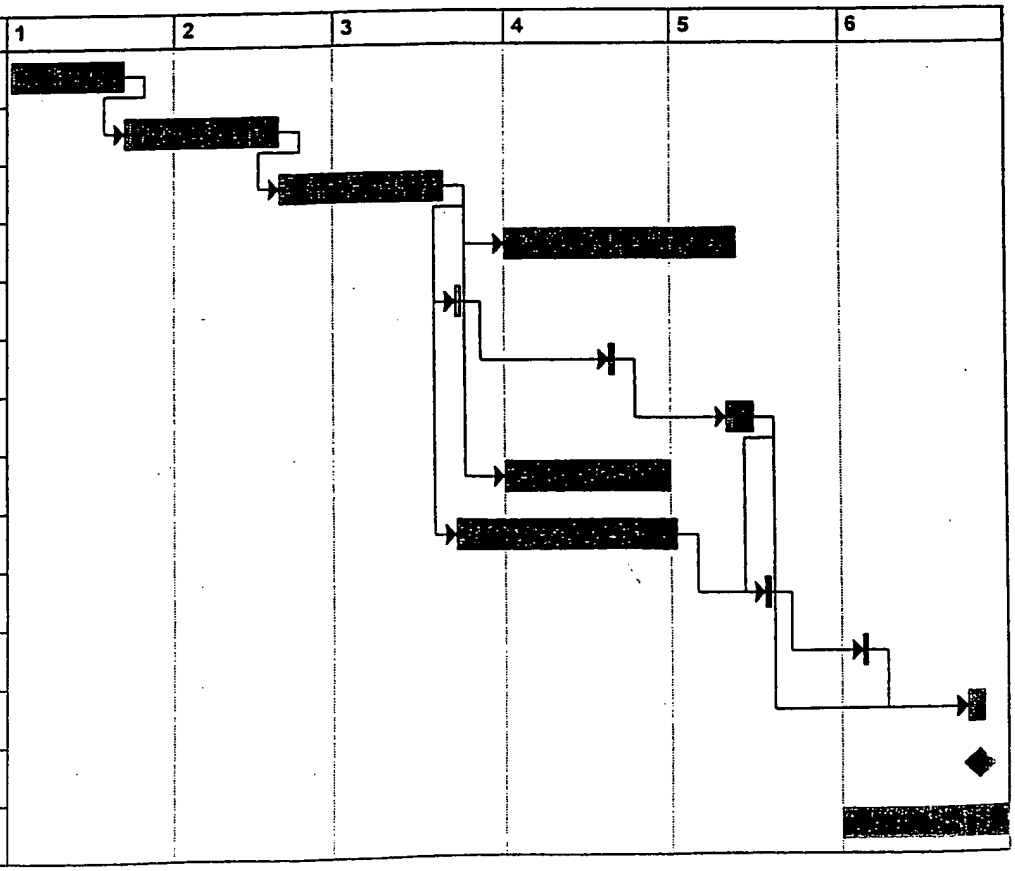
2. Yields of purified hybrid particles which exceed 50 mg/L, which will therefore minimize production costs.

3. Acceptable particle stability as determined by accelerated stability studies conducted at 37°C. Our goal is to develop a particle which is stable in solution for several months at ambient temperature.

4. Minimize the requirement for adjuvants beyond those currently approved (i.e. alum).

Timeline for the Completion of Phase I

ID	Task Name
1	Clone Univ. Malaria T-cell Site Onto HBc
2	Clone 3,6,9,12 NANP Repeats
3	Express and Purify Particles
4	Test Functionality of Th Epitope
5	Immunize Mice (3,6,9,12 Repeats)
6	Boost Mice (3,6,9,12 Repeats)
7	ELISA Analysis of Sera (3,6,9,12 Repeats)
8	Particle Characterization
9	Stability Studies
10	Immunize Mice (Stability)
11	Boost Mice (Stability)
12	Titer Comparison (Fresh v Incubated Particles)
13	Identification of Lead Vaccine Candidate
14	Write Phase I Report/Phase II Proposal



B. SIGNIFICANCE

Malaria

Malaria is by far the world's most important tropical parasitic disease, and kills more people than any other communicable disease, with the exception of tuberculosis. The causative agents in humans are four species of Plasmodium protozoa: *P.falciparum, P.vivax, P.ovale and P.malariae*. Of these, *P.falciparum* accounts for the majority of infections and is the most lethal.

- Malaria is a public health problem today in more than 90 countries, inhabited by a total of 2.4 billion people – 40% of the world's population.
- Worldwide prevalence of the disease is estimated to be on the order of 300-500 million clinical cases each year.
- More than 90% of all malaria cases are in sub-Saharan Africa. Two-thirds of the remainder are concentrated in six countries: India, Brazil, Sri Lanka, Viet Nam, Columbia and Solomon Islands, in decreasing order of prevalence.
- Mortality due to malaria is estimated to be in the range of 1.5 to 2.7 million deaths each year, accounting for one person every 12 seconds. The vast majority of deaths occur among young children in Africa, especially in remote rural areas with poor access to health services.
- The death rate of children below the age of 5 years is high but older children and adults generally survive, although they may have bouts of illness and may continually carry the organism.
- There are 7 million travelers from the U.S. each year to endemic areas. One source estimates that there are as many as 20 million arrivals from the U.S. into countries, other than Africa, where malaria is prevalent (World Tourism Organization).
- The U.S. reports about 1,000 cases of malaria each year. About 10,000 cases are reported each year in Europe, alone.

(Data from , WHO Factsheet #94, December 1996 and [44])

Figure 1: Malaria is a public health problem today in more than 90 countries, inhabited by a total of 2.4 billion people – 40% of the world's population.

Malaria infection begins when a person is bitten by a female *Anopheles* mosquito infected with one of the four *Plasmodium* species infectious for humans. The mosquito's saliva carries the malarial sporozoites into the blood. Approximately 30 minutes later these sporozoites enter the liver. Once in the liver, the sporozoites divide over the course of about 5 days, forming a schizont. A schizont may contain up to 30,000 merozoites which spill into the bloodstream the schizont ruptures. Within seconds, merozoites infect red blood cells (RBCs) and again replicate asexually, with each schizont producing up to 36 merozoites. Each time a RBC bursts and liberates progeny, other blood cells are infected. The cycle may continue until the

person dies of anemia and/or other complications. A few of the merozoites in RBCs differentiate into gametocytes, a sexual form, which, if ingested by a mosquito, are liberated from the RBCs in the mosquito stomach and subsequently mate. The progeny, sporozoites, accumulate in the saliva and the process starts again when the mosquito feeds (see 41 for review).

Malaria Prevention

Presently, there is no effective vaccine against malaria. For many years, **chloroquine** was a cheap and effective therapeutic for treating malaria, but in recent years chloroquine-resistance has increased dramatically. Indeed, a seven-fold increase in malaria in Senegal has been linked to emergence of chloroquine resistance, yet resistance in this country is much less prevalent than in other parts of Africa [44]. The same is true for newer anti-malarial drugs. Within just five years of mefloquine's arrival in Thailand, resistant parasites had emerged. In parts of Thailand and Cambodia, there are now parasites that are resistant to all the conventional drugs. Today, almost no endemic country is without drug resistant parasites. Moreover, because manufacturing such drugs tends to be unprofitable, fewer drug companies than ever before are developing new anti-malarials.

Perhaps surprisingly, one of the most effective methods for combating malaria in developing countries has been the use of **insecticide impregnated bed-nets**. Some scientists argue that such control methods – which have been shown to cut mortality and morbidity by 15 to 33 per cent in short-term trials – may in the long term only make populations more vulnerable to severe disease by reducing natural immunity. If they are correct, methods such as bed-nets and genetically engineered mosquitoes would have little impact on the control of the disease, and could indeed make matters worse [44].

Clearly, the most effective approach to combating malaria is an effective vaccine. As has been demonstrated with smallpox, a coordinated worldwide vaccination program can result in the eradication of communicable diseases

Malaria Vaccine Development

There are three recognized approaches to malaria vaccine development which are proposed to function by interrupting the parasite's lifecycle at three different stages.

The first, and most attractive approach, is the **pre-erythrocytic vaccine** which aims to block sporozoite entry into the hepatocyte and/or release of merozoites into the blood stream. Immediately following infection, sporozoites migrate to the liver and begin the exoerythrocytic stage of their lifecycle. An ability to block hepatocyte entry or the destruction of infected hepatocytes prior to liberation of merozoites would prevent the disease, the passage of the parasite on to feeding mosquitoes, and merozoite release and subsequent invasion of red blood cells.

A second approach is to develop an **'antidisease' vaccine**. This would not prevent infection, but it would block the body's harmful response to infection, which is thought to be responsible for many of the symptoms associated with infection.

A third approach, known as the **'altruistic' vaccine**, would not stop infection or symptoms in the individual but would prevent infection from spreading to others.

ICC's candidate malaria vaccine is a pre-erythrocytic vaccine - it aims to prevent infection by enabling the immune system to 'clear' the pathogen prior to the release of merozoites from hepatocytes (events up to this point are asymptomatic). A historical perspective of this type of malaria vaccine is given below.

A Brief Review of Pre-Erythrocytic Vaccine Candidates

The quest for a malaria vaccine was built on the observation that people repeatedly bitten by mosquitoes gradually acquire immunity over a period of many years [44]. Moreover, in the 1960s the British scientists Sydney Cohen and Sir Ian McGregor discovered that antibodies from such individuals could reduce the parasite load in people lacking immunity and clear the disease symptoms.

Around the same time, researchers at New York University (NYU) achieved full protection for the first time by injecting animals with small numbers of sporozoites from mosquitoes that had previously been irradiated. Later, researchers at the University of Maryland, NYU and Walter Reed Army Instiute showed that 90% of a group of human volunteers immunized with sporozoites from irradiated sporozoites later resisted exposure to virulent sporozoites [6, 7]. This confirmed that protective immunity to the sporozoite stage (i.e. the pre-erythrocytic stage) of the malaria parasite could be induced [45]. However, an inability to culture sporozoites *in vitro* thwarted the possibility of using sporozoites as a vaccine. Sporozoites still cannot be cultured *in vitro*.

The strategic development of a synthetic malaria vaccine required the identification of immunodominant/neutralizing malaria epitopes. In 1985, a group at NYU led by Drs. Ruth and Victor Nussenzweig, identified the dominant B cell epitope from the circumsporozoite protein (CS), a major component of the sporozoite surface membrane at the time the parasite enters the bloodstream [5]. Antibodies to this epitope (NANP)n were shown to be sporozoite neutralizing by protecting against rodent and human malaria [8]. Antibodies to the CS protein also appeared to correlate positively with protection in naturally infected individuals.

These studies leave little doubt as to the ability of anti-CS repeat antibodies to protect against malaria infection, providing sufficient antibody titers can be raised. The identification of this epitope therefore enabled the strategic development of synthetic CS-based malaria vaccines. Several vaccine candidates employing different carriers were developed based upon the identification of this epitope; a brief overview of four of them is given below.

1. Tetanus Toxoid Conjugated Synthetic Peptides

The (NANP)3 synthetic peptide, conjugated to the protein carrier tetanus toxoid (TT), was the first synthetic malaria vaccine to undergo phase I and phase II clinical trials in the late 1980's [9-11]. TT is widely known to provide powerful T cell help for coupled immunogens. Of the thirty-five vaccinees, the three having the highest titers of anti-sporozoite antibody were selected for challenge studies. One of the vaccine recipients remained free of parasitaemia at 29 days, while the other two did not exhibit asexual stage parasites until 11 days, compared with a mean of 8.5 days for the un-vaccinated control group. **Therefore, protection appeared to correlate positively with anti-NANP titers.**

The limited effectiveness of this vaccine was attributed to suboptimal levels of anti-NANP antibodies. Attempts to increase dosage were hindered by toxicity of the TT carrier. Further, the lack of parasite-derived determinants capable of priming malaria-specific T cells also likely contributed to the low levels of protection.

2. FSV-1

Short synthetic peptides often have an *in vivo* half-life which is too short for them to be effective as prophylactic or therapeutic drugs. Standard approaches for increasing the immunogenicity of peptides is to either couple them to larger carrier proteins, or to assemble them into multimeric structures. In this case 32 copies of the CS repeat sequence ((NANP)15(NVDP))2 were linked and produced recombinantly fused to a random 32 amino acid fusion protein [12]. This vaccine candidate was called FSV-1.

Upon immunization, twelve of the fifteen volunteers developed antibodies that reacted with sporozoites. No patients exhibited adverse reactions to the protein, indicating that the NANP repeat itself is non-toxic. Of the fifteen patients immunized with 3 doses, six were selected to receive a fourth dose and were then challenged with the malaria parasite. Parasitaemia did not develop in the volunteer with the highest titer

of CS antibodies, and parasitaemia was delayed in two of the other five vaccinees. **As with the NANP-TT vaccine discussed above, protection appeared to correlate positively with anti-NANP titers.** This vaccine was deemed partially successful in that it reconfirmed that humans can be protected by CS protein subunit vaccines. However, the level of protection was not sufficient to warrant larger trials of this particular candidate.

The major shortfall of this vaccine was that it did not provide an efficient source of T cell help. The only individuals who would have received T cell help from this vaccine would be those in whom the CS repeat serves as both a B and Th cell epitope. However, this sequence is known to be a Th epitope for only a limited number of individuals, i.e. it is highly genetically restricted. In an attempt to circumvent the genetic restriction of using the NANP repeat alone, Good, Berzofsky and colleagues identified a superior T_h epitope (326-343) on the CS protein outside the NANP repeat region, which appeared to be the immunodominant T cell site on the CS protein. When coupled to the NANP repeat, the T_h epitope did indeed appear to circumvent the genetic restriction observed when NANP alone was the immunogen [13]. This epitope formed the basis for the development of the universal T cell epitope to be discussed later.

3. Multiple Antigenic Peptides

Nardin and coworkers at NYU have been able elicit relatively high titers of anti-CS antibody in a diverse range of genetic backgrounds by combining the NANP repeat epitope with the T cell site identified by Berzofsky and Good [13] in a MAP format [14]. Using their proprietary 'universal' form of the CS-T cell epitope, Nardin and co-workers have been able to elicit anti-CS antibodies in all genetic backgrounds tested, suggesting that genetic restriction is alleviated by inclusion of this epitope.

While MAPs have proven to be excellent research tools, providing valuable insight into immune recognition of the CS protein, there are several intrinsic problems associated with using them in a commercial vaccine. Their commercial utility has yet to be established relative to manufacturing and cost issues.

4. DNA Vaccines

Over the past 5 years, significant progress has been made in the development of DNA vaccines which can potentially protect against malaria infection [42]. This class of vaccines appears to be particularly amenable to the efficient priming of CTL responses, although, as with all DNA vaccines, the exact mechanism by which this is achieved remains unresolved. In 1994, a group led by Dr. Steven Hoffman successfully protected 9 of 16 (56%) mice against *P.yoelli* infection using a DNA vaccine expressing the *P.yoelli* CS protein [15]. It was later shown that this vaccine protected only one of five strains of mice (H-2d) to a level of 75% [16]. A second DNA vaccine (PyHEP17) protected three of the five strains (H-2a, 71%; H-2k, 54%; H-2d, 26%) and the combination of vaccines protected 82% of H-2a, 90% of H-2k, and 88% of H-2d mice. Protection was absolutely dependent on CD8+ T cells, INF-gamma, or nitric oxide [16]. A human clinical trial using a similar multi-gene DNA vaccine designed to induce protective CD8+ T cell responses against *P. falciparum* infected hepatocytes was recently initiated [17, 18]. Initial results from these trials are expected sometime in 1998.

While there is only minor opposition to the use of gene therapy to treat life-threatening conditions such as cancer, its prophylactic use in healthy individuals remains controversial. These concerns are driven by the unknown long term effects of DNA immunization, such as the fear that the DNA may integrate into chromosomes to induce mutations and genetic abnormalities and the propensity for the production of anti-DNA antibodies associated with autoimmune diseases such as Lupus. There is already evidence that young children with certain predispositions could be at risk for certain forms of organ specific autoimmune problems [42]. For populations living in endemic areas, the benefits of protection against such a lethal disease as malaria may out-weigh the concerns associated with the unknown side-effects of DNA immunization. However, for travelers from North America and Western Europe, they may not.

While DNA-based therapeutics and prophylactics have clearly introduced an added dimension to modern medicine, many believe that they should not be regarded as a universal solution to all ailments. We are of this belief and remain convinced that parallel development of vaccines using alternative approaches is

essential when targeting a pathogen which continues to resist multiple vaccine strategies, almost 25 years after humans were first protected against malaria using irradiated sporozoites [6, 7].

5. HBsAg-CS

One of the most promising malaria vaccines of recent times utilizes the hepatitis B surface antigen (HBsAg) to deliver CS epitopes, an approach developed by SmithKline Beecham (SB) and colleagues. The CS epitopes include the NANP repeat, in concert with additional CS epitopes, including the T cell site identified by Berzofsky and Good [13] (but not the universal form developed by Nardin and co-workers [3, 4], fused to the hepatitis B surface protein [19]. This vaccine was recently the subject of human clinical trials [19]. When administered with one of three different adjuvants, this vaccine protected 1/7, 2/7 and 6/7 individuals respectively. Of the seven individuals immunized with vaccine 2 (adjuvant: oil-in-water emulsion), none of the five patients with anti-CS titers (IFA) in the range of 100-12,800 were protected, while the two vaccine recipients with antibody titers in the range of 25,600-51,200 were both protected. **Again, protection correlated with anti-CS titers.**

The strong reliance of this candidate vaccine on powerful immune-enhancing adjuvants was evident as they appear to be critical in compensating for its apparent low immunogenicity. For example, the enhanced protection to vaccine 3 (adjuvant: monophosphoryl lipid A and QS21), did not correlate well with antibody titers. This suggests that either CTL priming or non-specific immune stimulation may be playing a significant role in protection; if the latter is true protection may be short-lived. Patients were protected against malaria for 60 days, but no data has been presented on whether these patients remain protected beyond this period. This vaccine is currently undergoing field trials in the Gambia.

There are legitimate concerns as to whether such powerful adjuvants are too toxic for human use. Indeed, several participants of SB's clinical trial experienced "severe symptoms" after the second dose of vaccines 2 and 3 [19], while none of those receiving vaccine 1 (adjuvant: alum and monophosphoryl lipid A) exhibited such symptoms. These adverse systemic reactions "may have resulted from the intensity of the immune response after the second dose" [19]. However, toxicity of the adjuvant cannot be ruled out, particularly in light of the fact that no adverse reactions were noted with vaccine 1 (adjuvant: alum and monophosphoryl lipid A), and none are routinely observed with the existing hepatitis B virus vaccine which has a very similar composition. Unlike alum, the adjuvant QS21 has not been approved for human use and strong reliance on such adjuvants for vaccine efficacy may be a concern.

Table 1: Comparison of Primary Antibody Responses After Immunization with HBsAg and HBcAg.

Strain	H-2	Anti-HBs (Titer)	Anti-HBc (Titer)
B10	B	256	40,960
B10.D2	D	1024	81,920
B10.S	**S**	**0**	**163,840**
B10.BR	K	32	163,840
B10.M	**F**	**0**	**20,480**
B10.P	P	1024	10,240
C3H.Q	Q	2048	327,680
BALB/c	D	1024	327,680

Groups of mice of the indicated strains were immunized with a single dose (4μg) of either HBsAg or HBcAg and sera were examined for anti-HBs and anti-HBc antibodies 3 weeks later by ELISA) [46].

Like HBc, HBs is a particulate protein derived from the hepatitis B virus which has been proposed as a carrier for heterologous epitopes. We have studied the relative immunogenicity of HBsAg compared with HBcAg, and the ability of each to evoke immune responses in different genetic backgrounds (Table 1) [46]. These data emphasize both the higher immunogenicity of HBc relative to HBs, and the universal

responsiveness to HBc, irrespective of genetic background. For example, HBc is >300x more immunogenic than HBs in BALB/c mice; and, while both B10.S and B10.M mice are non-responders for HBs, every strain tested is responsive to HBc. These results re-emphasize the suitability of HBc as a vaccine carrier and specifically, its superiority over HBs, hence our selection of HBc as opposed to HBs to carry heterologous epitopes, and, in the case of malaria, overcome genetic restriction and inadequate antibody titers which have prevented the development of an effective vaccine.

Summary of Previous Pre-Erythrocytic Stage Malaria Vaccine

The positive correlation between protection against malaria infection and anti-CS antibody titer has been demonstrated repeatedly over the past 15 years [9-12, 19]. The evidence that a vaccine which can elicit high-titer, long-lived antibody responses in sufficient vaccine recipients can be protective suggests that protection against malaria infection is achievable via neutralizing antibody production. With delivery systems for the NANP epitope apparently exhausted, many groups have switched focus to alternative delivery systems, such as DNA and powerful adjuvants, in concert with the identification of new pathogen-neutralizing B and T cell epitopes from other stages of the parasitic lifecycle (discussed in "Phase II" section). While this work will increase the repertoire of reagents which can be used to develop effective malaria vaccines, we remain convinced that a delivery system that optimizes the interaction between the CS epitopes at hand, and the host immune system, will result in an effective malaria vaccine. **The high level of protection (90-100%) achieved with the HBc/CS-repeat is highly encouraging, and clearly warrants further testing of the *P.falciparum* homolog..**

The Role of Cytotoxic T cells in Pre-Erythrocytic Stage Immunity

The exact role of cytotoxic T cells in sterile immunity to malaria remains to be determined, although immunity elicited by irradiated sporozoites can be eliminated in some strains of mice by treatment with antibodies to $CD8^+$ T cells. The exoerythrocytic form of the parasite found in the hepatocytes has been identified as a target of this cell-mediated immunity [Hoffman, 1989 #648]. However, HBc particles expressing just the *P.bergeii* CS-repeat epitope afforded >90% protection against *P.bergeii* infection [1], suggesting that priming of CD8+ cells may not be essential for protection. Further, the successful protection of >90% of mice against *P.yoelli* with an HBc particle expressing only the *P.yoelli* CS-repeat tends to conflict with previous suggestions that cell-mediated immunity is necessary for protection in this rodent model, providing titers of neutralizing antibody are sufficiently high [2].

The contribution of T_c cells in vaccine trials involving irradiated sporozoites, or large portions of the CS protein which includes T_c epitopes such as SB's HBsAg-CS vaccine, remains to be determined. The strength of the HBc carrier technology is in the delivery of defined, linear B cell epitopes. Although HBc has been shown to prime pathogen specific T_h cells efficiently by independent groups [1, 20], HBc has not been shown to deliver foreign CTL epitopes, although a CTL response to HBc is elicited during the natural course of HBV infection [21, 22], and is the focus of new HBV vaccines [23-25]. These studies have shown that optimal CTL effects of HBc-CTL epitopes appear to require alternate deliver mechanisms, such as DNA [25, 26] and complex adjuvant formulations [23].

We believe strongly that there is sufficient data to suggest that immunity to malaria can be achieved through neutralizing antibody specific for the NANP repeat, providing that antibody titer is sufficiently high and long-lived in the majority of vaccine recipients. While we are confident that the HBc carrier, the NANP epitope and CS universal T cell site will afford such immunity, we remain cognoscente of the benefit of incorporating B and T cell epitopes from other malaria proteins expressed during other stages of the parasitic lifecycle, into our vaccine (see Phase II work).

Hepatitis B Core Protein

The hepatitis B virus core protein (HBc) is a 22kDa protein which spontaneously assembles into a particulate structure (27 nm) in the course of virion assembly during HBV infection and also during heterologous expression in both Prokaryotic and Eukaryotic systems [27]. Unlike the surface protein of the hepatitis B virus (HBs), HBc is highly immunogenic and immune responses to it show no significant degree of genetic restriction (see Figure 1) [46], making it an ideal candidate to serve as a carrier moiety.

In the late 1980s it was determined, using synthetic peptides, that the major immunodominant B cell epitope on HBc is localized around amino acids 75-83 [47; Milich and Thornton, unpublished observation]. Since the identification of this site, several research groups have successfully cloned foreign epitopes into this immunodominant loop and successfully demonstrated that HBc is indeed a unique and highly effective carrier moiety for B cell epitopes[27]. The multimeric, particulate nature of the assembled HBV nucleocapsid confers this enhanced immunogenicity to foreign sequences. Experimental examination of a variety of B cell epitopes, either chemically linked or fused by recombinant methods to HBc particles, has yielded significant success [27].

Earlier this year, two research groups based at the NIH and the MRC in the UK, solved the structure of HBc particles using cryo-electron microscopy (see Figure 2) [28, 29]. For those familiar with the immunological properties of HBc, it was not surprising to learn that the region encompassing amino acids 75-83 does indeed form a loop at the surface of the particle. Specifically, amino acids 78-82 appear to form a loop that connects adjacent helices (Figure 2B). Preliminary x-ray diffraction data is consistent with these predictions (R.Crowther - MRC, Seminar given at the Scripps Research Institute, Oct.1997).

HBc as a Carrier of Heterologous Epitopes

The hepatitis B core protein is one of the most immunogenic proteins known. In its natural state it forms the core of the hepatitis B virus and the immune system of essentially every individual who becomes infected with the virus develops a powerful immune response to the core [30].

Recombinantly produced, hybrid hepatitis B core proteins have been shown to significantly enhance the immune response to inserted foreign sequences displayed at the surface with a conformation that mimics the structure of the sequence in the parent protein [27]. The variety of hybrids produced in this manner have proven HBc to be amazingly tolerant to the internal addition of foreign sequences at the site of the immunodominant loop, while retaining the ability to form particles that can be easily purified in a manner that is largely independent of the inserted sequence.

As a carrier moiety for B cell epitopes HBc offers other significant benefits. The particulate structure stimulates immunogenicity and allows incorporation of multiple epitope copies that are conformationally constrained. These features confer superior results relative to non-particulate carriers such as KLH, BCG, or tetanus and diphtheria toxoids [31]. Further, HBc can directly activate B cells, elicit strong T_h cell responses, and is efficiently processed and presented by antigen presenting cells (unpublished data, D.Milich personal communication, 1997).

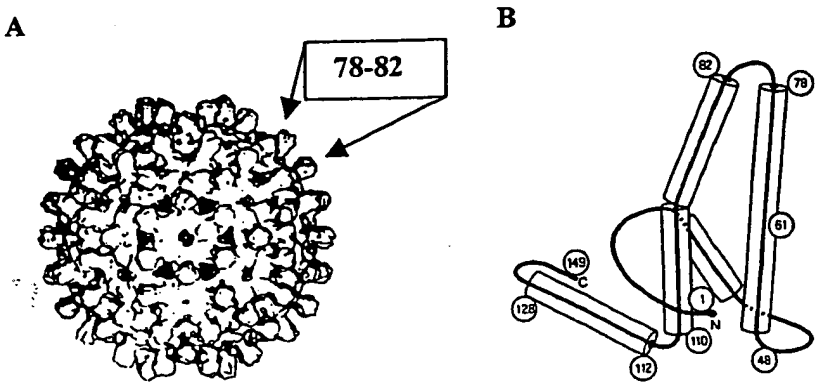


Figure 2: A: HBc particle structure determined at 9 angstrom resolution from cryo-electron micrographs. The immunodominant loop (78-82) is indicated. (From: Conway *et al.*, (1997) Nature 386, 91-94. **B: Proposed polypeptide fold for HBc.** Alpha-helical regions are shown as cylinders and the putative N- and C-termini are marked. An approximate numbering scheme for amino acids is given (From Bottcher *et al.*, (1997) **Nature 386**, 88-91)

A number of pathogen-related B cell epitopes have been chemically linked, or fused by recombinant methods to HBc, as a method to increase immunogenicity (HBV, HIV-1, FMDV, HRV-2, BLV, FeLV, HCV, MCMV, PV-1, SIV) (see 27 and 32 for review). **These studies, conducted by a number of independent laboratories, have yielded significant success; including complete protection against FMDV using a HBc/FMDV particle which elicited neutralizing antibody titers comparable to those observed following exposure to the FMD virus [33]. This work proved that neutralizing epitopes, presented in the context of HBc, can elicit protective (pathogen neutralizing) immunity.**

Using rodent malaria models, ICC's founding scientists successfully demonstrated that malaria CS-repeats fused to the immunodominant loop of HBc were able to protect mice against both *P.bergeii* and, prehaps more impresively, *P.yoelii* to levels of 90-100% [1, 2]. Further, antibody responses to the *P.bergeii* particle were shown to prime antibody responses effectively in a wide range of genetic backgrounds, confirming the universal priming effects of HBc (Table 2) [1].

Another advantage of the HBc carrier is the fact that it does not require complex adjuvants for efficacy. This is due to the inherent high immunogenicity of the particle. A comparison of the immunogenicity of HBc-CS1 particles showed that alum, which is approved for human use, was more effective than either IFA or CFA (Table 3) [Schodel, 1994 #682]. The importance of this observation is highlighted by toxicity problems associated with newer, more complex adjuvants as was recently noted in clinical trials of SB's candidate malaria vaccine [19].

Table 2: Immunogenicy of HBcAg-CS1 Particles in Mice.

Strain	H-2	HBcAg	CS	[DP4NPN]
C57BL	b	655,360	163,840	>655,360
B10.S	s	163,840	163,840	>655,360
B10.M	m	10,240	10,240	163,840
B10.BR	k	10,240	10,240	655,360
BALB/c	d	40,960	40,960	>655,360

Groups of three mice of the indicated strains were immunized with 20 µg of HBc-CS1 particles in CFA and boosted with 10 µg in IFA. Sera were collected before immunization,2 weeks after secondary immunization. Sera were pooled and analyzed by solid-phase ELISA: HBcAg (50ng/well); CS represents *P.bergeii* sporozoites (1,000/well); and the P.bergeii repeat [D4NPN]2 (1.0 ug/well) were the solid phase ligands. [1] (N.B. sporozoites were limiting for CS titer determination; Florian Schodel,, personal communication).

Table 3: Effect of Adjuvant on Immunogenicity of HBcAg-CS1 Particles.

Immunogen	Adjuvant	Time weeks	Anti-[DP4NPN]
HBcAg-CS1	CFA	2	10,240
		4	163,840
	IFA	2	2,560
		4	163,840
	Alum	2	10,240
		4	655,360

Groups of three BALB/c mice were immunized with HBcAg-CS1 particles (10 µg) prepared in three different adjuvants; CFA, IFA or alum. Sera were collected before immunization and 2 and 4 wk after primary immunization. Sera were pooled and analyzed by solid-phase ELISA using [DP4NPN]2 (1.0 µg /well) as solid-phase ligands. [1]

The B cell Epitope, (NANP)n

The immunodominant B cell epitope of the CS protein of *P.falciparum* is a highly conserved, repetitive tetrapeptide (NANP) [5], and antibodies to this epitope have been shown to be sporozoite neutralizing by protecting against rodent and human malaria. Immune responsiveness to this epitope has been positively correlated with immunity to malaria in both vaccine recipients and naturally infected individuals. Indeed, a review of clinical trials data for pre-erythrocytic vaccines described previously (HBs-CS, FSV-1, NANP-TT) highlights strong correlation between antibody titer and protection [9-12, 19]. Those individuals who have been protected by previous vaccine candidates are those with the highest anti-NANP antibody titers, with the possible exception of SKB's candidate vaccine (# 3) where adjuvants appeared to play a critical role in protection [19].

The Company believes that HBc is indeed the 'superior carrier' which will enable the development of high titer, long-lived antibody responses in vaccine recipients. Further, we believe that inclusion of epitopes which enable malaria specific T_h function will significantly enhance the potency and specificity of the vaccine.

In 1993 ICC founding scientists reported the protection of mice against *Plasmodium bergeii* infection using a vaccine constituting the *P.bergeii* repeat presented at the surface of HBc particles (HBcAg-CS1) [1]. More recently protection against *P.yoelli* has been described using a similar approach [2]. Initial evaluation of a particle displaying epitopes from P.falciparum were encouraging, but antibody titers in mice were lower than those observed for the *P.bergeii* and *P.yoelli* particles.

Table 4: Comparison of Immunogenicity of HBcAg-CS2 and HBcAg-CS2.1 in Mice. [1]

			Antibody Titer (1/dilution)	
Immunogen	Strain	Time (days)	HBc	[NANP]
HBcAg-CS2	B10	10	10,240	2,560
		24	40,960	10,240
		Secondary	655,360	655,360
	B10.S	10	10,240	640
		24	40,960	1,280
		Secondary	>655,360	20,480
	BALB/c	10	10,240	0
		24	40,960	640
		Secondary	>655,360	40,960
HBc-Ag-CS2(new)	B10 x B10.S F1	10	1,280	5,120
		24	10,240	40,960
		Secondary	163,840	2,621,440

Groups of three mice each of the indicated strains were immunized with 20 µg of hybrid HBc-CS2 or HBc-CS2.1 particles in CFA and boosted with 10 µg in IFA. Sera were collected before immunization, 10 and 24 d after primary and 2 wks after secondary immunization. Sera were pooled and analyzed by solid-phase ELISA: HBcAg (50 ng/well); the P.falciparum repeat [NANP]5 (1.0 µg/well). [1]

Scientists at ICC have recently improved the immunogenicity of the *P.falciparum*-HBc particle by altering the insertion strategy of the NANP repeat epitope at the surface of the HBc particle (HBc-CS2(new)) (proprietary data). This approach has yields a more immunogenic particle and evokes antibody titers which approach those achieved for the protective *P.bergeii* particle (Table 4) [1]. We are unaware of anti-NANP titers of this magnitude being achieved following a 2 dose immunization

regime. In addition to increasing the immunogenicity of the NANP repeat, we have simultaneously reduced the HBc-immunogenicity (Table 4). A direct comparison of the two particles, using identical mouse strains, is currently underway. The poor response of CS2 in BALB/c mice is somewhat puzzling since the anti-HBc response was very high and HBc does not have a history of exhibiting genetic restriction. However, we anticipate that this can be overcome during phase I work by optimizing the presentation of the NANP repeat on the HBc particle using the new presentation strategy and the inclusion of the malaria specific universal Th epitope which is highly effective in this strain (described below) (Table 7).

The Malaria Specific T cell Epitope, CS 329-345

While the T cell help afforded by HBc is highly effective in enhancing antibody responses (i.e. B cell mediated) to 'carried' epitopes following vaccination, it will not activate malaria specific T cells. To ensure the priming of malaria specific T-helper cells as well as B cells, we will include a malaria specific T-helper epitope in our vaccine.

Table 5: Comparison of Proliferative Responses of T cell Clones after in vitro challenge with peptide 326-345 variants of the *P.falciparum* CS Protein.

Variation in Sequence 326-345	Response				
	DR9-CA2F9	DR7-RMB11	DR7-RM2B10	DR4-DW2F9	DR1-RM1B10
EYLNKIQNSLSTEWSPCSVT	+++	+++	+++	+++	+++
K K	+++	++++	+++	+++	-
K KR	+++	+++	+++	+++++	-
K KT	++++	+	+++	-	-
K KT K	+	-	+	-	-
K Q	++++	++++	++	++	-
K Q K	++	+++	+	-	-
K Q R	+	+++	-	-	-
Q K	++++	++++	++	+++	-
Q KT K	+	++	+	-	-
Q K K I	++	++	++	-	-

The data are presented as percentage of stimulation obtained with 1ug/ml control peptide NFS4 326-345. -, <20%; +, 20-49%; ++, 50-79%; +++, 80-120%; ++++, 120-200%; +++++, >200%. After in vitro challenge with peptide 326-345 at 1ug/ml the stimulation index for DR9-CA2F9 was 82, for DR7-RMB11 was 30, for DR7-RM2B10 was 60, for DR4-DW2F9 was 6, and for DR1-RM1B10 was 47. [4]

The benefits of the inclusion of a universal T_h cell epitope derived from the malaria parasite are several-fold. First, the priming of malaria specific T_h cells ensures that, should a vaccine recipient be exposed to malaria, a more rapid and stronger anti-malaria response will be activated due to previous priming of malaria specific T-helper cells. Secondly, vaccinees living in malaria endemic regions will experience natural 'boosting' every time they are exposed to the parasite because their immune systems have been primed at both the B and T_h cell level. This effect is similar to clinical boosting by re-vaccination – a process which would be difficult to enforce in third-world countries where malaria is often endemic.

While the CS gene is largely invariant, limited sequence variation has been noted to occur mainly in the immunodominant T cell epitopic domains. The fact that genetic mutations always appear to result in amino acid substitutions suggests that pressure at the protein level, possibly immunological pressure, has selected for variation. Typically, the problems associated with amino acid variability of an epitope can only be resolved by the inclusion of multiple variants of the epitope. However, Nardin and coworkers at NYU recently identified a consensus form of the T cell epitope CS 326-345 which appears to bind to all class II molecules [3, 4]. Studies have shown that this consensus epitope is 'universal', like the T cell help afforded by HBc, and suggests that it will prime malaria specific Th cells in essentially all vaccine recipients (see Table 5). The fact that this epitope of the CS protein was identified by CD4+ T cells of volunteers protected against malaria following exposure to irradiated sporozoites confirms that this epitope is efficiently processed

and presented *in vivo* by antigen presenting cells (APC) when presented in the context of sporozoites [34]. The identification of this epitope was a significant advancement in the task of developing a pre-erythrocytic stage malaria vaccine, and it is this 'universal' T cell epitope that we are incorporating into our pre-erythrocytic stage malaria vaccine.

Table 6: Mapping of Epitopes within Amino Acid Sequence 326-345 recognized by T cell Clones Restricted by Different DR Alleles.

Amino Acid Sequence	Stimulation Index											
	DR9-CA2F9			DR7-RM2B10			DR4-DW2F9			DR1-RM1B10		
	10[a]	1	0.1	10	1	0.1	10	1	0.1	10	1	0.1
EYLNKIQNSLSTEWSPCSVT	112	76	27	200	169	88	122	29	25	43	3	1
EYLNKIQNSLSTEWSPCS	148	114	62	226	165	53	114	9	1	93	1	1
EYLNKIQNSLSTEWSP	130	98	23	205	167	75	35	2	1	1	1	1
EYLNKIQNSLSTEW	134	102	42	1	1	1	94	9	2	1	1	1
EYLNKIQNSLST	5	1	1	1	1	1	4	1	1	3	1	1
LNKIQNSLSTEWSPCSVT	114	75	26	220	159	53	1	1	1	1	1	1
KIQNSLSTEWSPCSVT	50	6	3	196	109	8	1	1	1	1	1	1
QNSLSTEWSPCSVT	4	4	1	162	14	1	1	1	1	2	1	1
SLSTEWSPCSVT	1	1	1	189	190	82	1	1	1	1	1	1
STEWSPCSVT	10	4	3	7	1	1	1	1	1	1	1	1

Cloned T cells (2 x 10 4) were cultured with irradiated autologous PBL (5 x 10 4) in the presence of various peptide concentrations ranging from 0.1 to 10 μg/ml. IL-2 levels were measured in 24-h supernatants using a bioassay based on proliferation of an IL-2 dependent T cell line.[b] Peptide concentration (μg/ml). [4]

Table 7: Genetic Restriction of Response to MAP Containing Universal T_h Epitope.

Responder	Strain	H-2	ELISA Titer
High	BALB/c	D	248,335
	DBA/1	Q	137,772
Intermediate	C57BL/10	B	57,926
	RIIIS	R	34,443
	A/J	A	23,525
Low	SJL	S	4,302
	C3H	K	3,620
	P/J	P	1,280

Results shown as geometric mean titers in sera obtained 20 days after the third injection of MAP using homologous MAP as Ag in ELISA. [4]

This epitope has also been shown to contain CTL epitopes recognized by human $CD4^+$ CTL [4, 34, 35] as well as human $CD8^+$ T cells [36] and, thus, may represent a universal T cell epitope, not only in terms of MHC binding but also in terms of immunologic function. Preliminary studies have shown that MAPs containing this epitope can elicit class-I restricted T cell responses in mice [4].

Production Issues

The initial failure of simple vaccine approaches to yield an effective vaccine against malaria has resulted in the development of increasingly more elaborate vaccine candidates, such as multiple antigenic peptides (MAPs), DNA vaccines, and engineered IgG fragments. While these approaches have shown promise, they do pose production complications, and in some cases safety concerns. Further, more elaborate vaccines are often so expensive that even if they are successful they are cost-prohibitive for commercial use,

particularly in the developing world where the vaccine is needed most.

In addition to the many functional advantages of HBc over other carriers, HBc particles are produced recombinantly in a highly cost-effective *E.coli* expression system. The use of a single DNA clone to produce a recombinant protein ensures absolute uniformity and reproducibility which cannot always be assured by chemical approaches which are often restricted by uncontrollable 'side-reactions'. Further, we anticipate that the immunogenicity enhancing properties of HBc will enable a 2 dose immunization regime for this vaccine.

Production of recombinant proteins in *E.coli* is a highly cost effective process. Presently we are able to produce in excess of 100mg of highly purified HBc/NANP particles (>98% by SDS-PAGE) from a 1L fermentation performed in a shaker flask. We are confident that yields can be improved significantly through the use of more efficient fermentation conditions. All purification procedures used to date at the laboratory scale are simple and scalable, permitting large quantities of material to be made and purified economically. The Company will out source all manufacturing of materials for toxicological and clinical testing to PrimaPharm in San Diego, CA. PrimaPharm possesses 20 years of cGMP manufacturing expertise and has the capability of producing, purifying, and vialing material for the malaria vaccine studies. It boasts major multinational pharmaceutical companies among its clients, including SmithKline Beecham and Allergan, and offers competitive pricing which will ensure that the final price of the vaccine will be highly competitive. An estimation of the cost for a 150L fermentation, plus purification and vialing, has been determined to be $17,000. A chart to show the production cost for 20μg doses of recombinant HBc/NANP particles is presented in Table 8. PrimaPharm is located approximately 100 yards from ICC's research labs which will enable their production staff to work closely with ICC's research and development team during the scale-up process.

Table 8: Costing of ICC's malaria vaccine based on a 150L fermentation, purification and vialing at a cost of $17,000. Yields of HBc-CS2(new) from a 1L culture grown in a shaker flask is typically 80-120 mg of purified particles. We are confident that yields closer to 200mg/L will be achievable with a production scale fermenter where pH, temperature, aeration and nutrient supply are more effectively controlled. The hepatitis B vaccine (HBsAg) is administered at doses of 10-20μg.

Yield (mg/L)	Yield from 150L	20μg Doses	Cost Per 20μg Dose
25	3,750 mg	187,500	9.1c
50	7,500 mg	375,000	4.5c
100	**15,000 mg**	**750,000**	**2.3c**
200	30,000 mg	1,500,000	1.2c

While toxicological studies of HBc-based products have not been conducted to date there is no evidence of toxicity due to HBc in HBV asymptomatic chronic carriers or in HBcAg-expressing transgenic mice.

Relationship with Future Research and Development

In addition to key *P.falciparum* epitopes, ICC is also in the process if licensing neutralizing CS-epitopes specific to other strains of human malaria. *P.vivax* is the second most important malaria target, and we intend to focus on the development of a *P.vivax* vaccine starting in 1999. The multimeric nature of the HBc particle means that it will be possible to assemble a multivalent malaria vaccine displaying neutralizing epitopes from both *P.vivax* and *P.falciparum,* either by co-expressing different HBc genes [37], or by inserting multiple epitopes into a single HBc gene. Multivalent vaccines are highly desirable as they enable costs, from production through vaccine administration, to be reduced significantly.

ICC is also actively involved in identifying additional human and veterinary vaccine targets using identified, neutralizing, linear epitopes which are amenable with presentation by HBc. We expect to identify our next 2 targets by the 2nd quarter of 1998 and begin pre-clinical development shortly thereafter. Our

experiences with the development of a HBc-malaria vaccine has already afforded us with additional information on to select candidate epitopes and expedite the development of HBc hybrid particles at the research level. We anticipate that this commonality in products will be even more important once we advance to the production stage and on to clinical testing.

Phase II

The phase II section of this proposal will focus on the clinical development of the vaccine once the lead has been identified at the end of phase I. Our clinical procedures will follow the "Guidelines for the Evaluation of Plasmodium Falciparum Vaccines in Populations Exposed to Natural Infection" distributed by the World Health Organization. The time-line for clinical development of the malaria vaccine is outlined below.

4th Quarter 1998	Identify Lead Compound (Phase I)
4th Quarter 1998	Identify Clinical Sites
1st Quarter 1999	Develop Manufacturing and Purification Methods for cGMP Material
2nd Quarter 1999	Complete GLP/GMP Manufacturing of Vaccine
3rd Quarter 1999	Complete Toxicological Studies
4th Quarter 1999	Submit IND
1st Quarter 2000	Phase I/II Clinical Trials

At a research level, work on the development of a next generation malaria vaccine will continue during phase II by improving the immunogenicity of the *P.falciparum* particle via the inclusion of addition epitopes. Indeed, ICC is in the process of establishing collaborative arrangements with academic groups to focus on the identification of such epitopes, including those derived from proteins expressed during liver and blood stages of the parasite's lifecycle. Potential candidates include the merozoite surface protein-1 (MSP-1), which is considered a 'blood stage antigen'. Epitopes within MSP-1 are the target of antibodies which inhibit erythrocyte invasion *in vitro* [Blackman, 1990 #484; Chang, 1992 #258; Cooper, 1992 #2309; Hui, 1994 #716], and are particularly promising candidates because they show no amino acid variation. Vaccination experiments with the equivalent polypeptide from *P.yoelii* is protective [38, 39], and protection appears to be mediated largely by antibody [38, 40], making it an ideal candidate for presentation by HBc once neutralizing epitopes have been more precisely mapped. At the present time, these epitopes are poorly defined beyond the clear importance of the highly disulfide bonded C-terminal 19kDA region of MSP-1 [Ohta, 1997 #2134; Egan, 1997 #2405; Guevara Patiño, 1997 #2408]. Once new, pathogen neutralizing epitopes derived from MSP-1, or other target proteins which are amenable with the HBc presentation strategy have been identified, they will be tested.

Key Patents Held By ICC

ICC has exclusive or semi-exclusive licenses to the following patents which are relevant to this work:

1. T Cell Epitopes for the Hepatitis B Virus Nucleocapsid Protein (Patent # 5, 143,726)
Inventors: Thornton, Moriarty, Milich, and McLachlan.
Immune Complex Corporation holds an exclusive license of this patent from The Scripps Research Institute, La Jolla, CA.

2. Immune Complex Corporation is in the final stages of negotiating exclusive and non-exclusive licenses to issued patents and patent applications describing critical malaria B and T cell epitopes necessary for the development of this vaccine. The final terms are scheduled to be completed by January 31, 1998. At the time of review, confirmation of this arrangement will be available upon request.

Potential For Commercial Application

With 7 million U.S. citizens traveling to malaria endemic areas, the initial retail market for the vaccine in the U.S. alone is over $1.1 billion dollars annually (CDC, Harvard School of Public Health). The market size in dollars for purchases by point of care for a 50% market share will be closer to $250 million annually.

In addition to the US travelers market, the market in dollars of travelers from other developed countries to malaria endemic areas is thought to be at least twice that of the U.S. market or another $500 million. A total of 30-35 million people from developed countries travel to less developed ones, many of which are in the tropics. At least 30 % of those succumb to illness [47]. As many as one in every 100 people from industrialized countries who visit West African countries will get malaria; those who visit rural areas, away from the relative safety of air-conditioned urban hotels, are the most likely to become infected.

Sales to the World Health Organization at a largely reduced price but high volume is estimated at $20 million annually (60c per dose; Howard Engers, personal communication with Ben Thornton, 1997). Malaria is endemic in 91 countries. Eighty percent of the cases occur in Africa where it accounts for 10% to 30% of all hospital admissions. Malaria, thus, is a heavy economic burden on developing countries. The cost of treatment is between $US 0.08 and $US 5.30 depending on the drugs prescribed. The total cost of malaria in terms of health care, treatment, lost production, etc. is estimated to be $US 1.8 billion. For example, India will spend U.S. $40 million on malaria control this year – up 60 % from last year. It is also planning a five-year, $200 million program targeting 210 million people in 100 high-risk districts. The program will introduce new tools such as pesticide-impregnated bednets – a much less-effective preventative measure compared with an effective vaccine [44].

Although other vaccines are currently being developed by other pharmaceutical companies, the Company believes that its HBc-based malaria vaccine will have economic, compliance, and efficacy advantages over its competition.

B. RELEVANT EXPERIENCE: PI, STAFF, and CONSULTANTS

Ashley J. Birkett, Ph.D. (Principal Investigator)

Dr. Birkett has extensive experience working with the hepatitis B core protein. He is particularly familiar with the use of hepatitis B virus core protein (HBc) as a carrier of foreign peptide sequences. These include the insertion of foreign epitopes for raising polyclonal antibody, expression of short proteins as C-terminal extensions of HBc, and antigenic/immunologic characterization of hybrid HBc particles. Dr. Birkett is the recipient of a phase I SBIR grant to study the chemical insertion of synthetic epitopes into HBc using native chemical ligation of protein fragments (Title: Epitope Scanning Using Protein Semisynthesis, Grant # 1-R43-GM54461-01), and is in the process of filing multiple patents to cover the novel uses and significant improvements he has made to the HBc carrier system. He has also worked extensively on a NIEHS contract (Contract # NIH-ES-52002, PI Dr. H.J.Barnes), currently in phase II, to use HBc as a carrier for generating high affinity, inhibitory antibodies to human cytochrome P450s for use in *in-vitro* testing of candidate therapeutic drugs by drug discovery groups.

Ph.D. Biochemistry (1994)
Medical College of Virginia/Virginia Commonwealth University
Richmond, Virginia.
B.Sc. (Honors) Applied Biological Sciences (1990)
Bristol Polytechnic, Bristol, UK.

Immune responses to the hepatitis C virus NS4a are profoundly influenced by the combination of the viral genotype and the host major histocompatibility complex. Zhang Z-X, Chen M, Hultgren C, **Birkett A**, Milich DR, Sällberg M. (1997) *J Gen Virol.* 78(Pt 11):2735-2746.

Interferon-alpha treatment induces delayed CD4+ proliferative responses to the hepatitis C virus non-structural 3 protein regardless of the outcome of therapy. Zhang Z-X, Milich DR, Peterson DL, **Birkett A**, Schwarcz R, Weiland O, Sällberg M. (1997) *J Infect Dis.* 175(6):1294-1301.

Cloning, Expression, Purification and Characterization of the Major Core Protein (p26) from Equine Infectious Anemia Virus. **Birkett, A.J.** , Yélamos, B., Gavilanes, F., and Peterson, D.L. (1997) *Biochim Biophys Acta*, 1339(1):62-72

Antibody production to the hepatitis C virus core and non-structural 3 proteins is highly sensitive to deficits in T cell function. Ando Y, Sönnerborg A, Barkholt L, **Birkett A**, Ericzon BG, Sällberg M. (1997) *Clin. Diagn. Lab. Immunol.* 4:104-106

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Use of N,N, Dimethyl Lysine Peroxidase in Immunoassays. Jin, L., Wei, X., Datta, M., Gomez, J., **Birkett, A.**, and Peterson, D.L. (1995) *Anal. Biochem.* 229:54-60.

Determination of Enzyme Specificity in a Complex Mixture of Peptide Substrated by N-terminal Sequence Analysis. **Birkett, A.J.**, Soler, D.F., Wolz, R.L., Bond, J.S., Wiseman, J., Berman, J. and Harris, R.B. (1991) *Anal. Biochem.* 196:137-143.

Evidence for the *in vivo* Deamidation and Isomerization of an Asparaginyl Residue in Cytosolic Serine Hydroxymethyltransferase. Artigues, A., **Birkett, A.** and Schirch, V. (1990) *J. Biol. Chem.* 265:4853-4858.

George B. Thornton, Ph.D. (President/CEO)

Dr. Thornton joined the ICC/Synthetic Genetics in 1996, becoming the President and CEO in August. He was formerly the Vice President of Research and Development at GeneMedicine (Houston), a gene therapy biotechnology company developing non-viral approaches to gene delivery. While at GeneMedicine he was responsible for all research activities and the development (preclinical and clinical) of their gene therapy products. Two GeneMedicine programs directed by Dr. Thornton have received phase I approvals this year. Prior to his position at GeneMedicine, Dr. Thornton was Director of Research at the Johnson & Johnson PRI laboratories in La Jolla where he was responsible for research and operations at that site. While at Johnson & Johnson, scientists lead by Dr. Thornton were the first to successfully obtain a human antibody derived from phage displaying human antibody combinatorial libraries. In addition, he set up and was responsible for the J&J laboratories in Sydney, Australia which was developing gene therapy products based on ribozyme technologies licensed from the CSIRO (Canberra). The hepatitis B core technology was developed as a collaboration between Dr. Thornton's laboratory at J&J and Dr. Milich's laboratory at The Scripps Research Institute. Dr. Thornton is a Founder of the Company and co-inventor on the Company's key hepatitis B core patents.

D. EXPERIMENTAL DESIGN AND METHODS

Cloning of Universal Malaria T_h Epitope onto HBc

In the hepatitis B virus, the HBc protein is 183 amino acids in length. Carboxyl-terminal truncations of HBc to 144 are amenable with particle assembly, and are generally preferable since amino acids 150-183 encodes an arginine-rich domain which binds viral nucleic acid. When expressed in *E.coli* this domain non-specifically binds *E.coli* mRNA. The 20 amino acid universal T_h epitope will be inserted after amino acid 149 of HBc using PAGE purified complementary synthetic oligonucleotides, designed to have an *Eco*RI single strand overhangs at the 5' end, and a *Sac*I overhangs at the 3' end precluding prior restriction digestion of the insert. However, the synthetic fragment will NOT restore either restriction site as it is necessary to destroy the restriction sites for later cloning steps. Once annealed, this fragment will be directionally ligated into the plasmid pKK-HBc149-CF which has *EcoRI* and *SacI* restriction sites positioned directly after amino acid 149 of HBc. Clones will be identified by restriction endonuclease mapping, and then confirmed by DNA sequencing.

Generation of HBc/NANP Hybrid Particle Expression Vectors

To construct the hybrid HBc-(NANP)n expression vectors complete with the universal malaria T_h epitope at the C-terminus, it will be necessary to adopt a 2-step cloning strategy. First, PAGE purified synthetic complimentary oligonucleotide fragments encoding for (NANP)3, (NANP)6, (NANP)9, and (NANP)12 repeat epitopes will be annealed and directionally ligated into the cloning/expression vector pKK322-HBcLI. This vector *has EcoRI* and *SacI* restriction sites introduced between amino acids 77 and 78 which is the middle of the immunodominant HBc loop. Again, pairs of oligonucleotides will be designed to have *Eco*RI single strand overhangs at the 5' end, and a *Sac*I overhangs at the 3' end precluding prior restriction digestion of the insert. However, the restriction sites will be restored after cloning of the insert. The second step will be to incorporate the universal T_h epitope exchanging the C-terminal fragments of HBc

for the fragment with a fragment from which also contains the universal Th epitope. All constructs will be verified by restriction analysis and sequencing prior to expression.

Expression and Purification of HBc/NANP Hybrid Particles.

Once constructed, expression will be transformed into *E.coli*, strain TB1. Cells will be grown for 36 hour in TYN media supplemented with 1 g/L glucose and harvested by centrifugation. To purify the hybrid particles, cells from a 1L culture will be lysed in PBS by a single passage through a French pressure cell. Following clarification of the lysate, the particles will be precipitated by ammonium sulfate, resuspended in 20 mM sodium phosphate, pH 6.8, and dialyzed against the same buffer. The dialyzed material will be clarified by a brief centrifugation and the supernatant subjected to gel filtration chromatography on a Sepharose CL-4B column (see Figure 3). Particle containing fractions will be identified immunologically, subjected to hydroxyapatite chromatography, and reprecipitated by ammonium sulfate prior to resuspension, dialysis, and sterile filtration prior to storage at –70°C. Typical yields of HBc-NANP particles, from a 1L shaker flask culture, are in the range of 80-120 mg of purified particles.

Physical Characterization of Hybrid Particles

HBc hybrid particles have a characteristic elution position when analyzed by Sepharose CL-4B chromatography (see Figure 3). In addition to Sepharose CL-4B chromatography, the production of uniform hybrid particles will also be confirmed by electron microscopy (see inset on Figure 3).

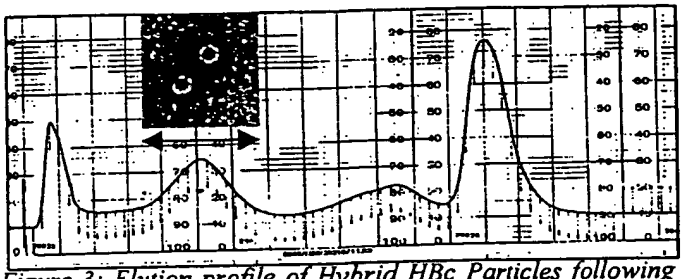

Figure 3: Elution profile of Hybrid HBc Particles following Sepharose CL-4B Chromatography. Arrow indicates elution range of HBc particles. Inset: Electron Micrograph of Hybrid HBc Particles.

Immunization of Mice with Hybrid Particles

Mice will be immunized with 20 µg of hybrid particles in alum, and boosted 28 days later with 10 µg of hybrid particles in alum. Mice will be bled and antibody titers measured 2 and 4 weeks post-primary immunization, and 3 weeks post boost. To determine if there is MHC-specific genetic restriction, the following mouse strains will be immunized B10(b), B10.S(s), B10.P(p), B10.D2(d), and B10.BR(k).

ELISA Testing of Anti-NANP Antibodies

Antisera will be evaluated using (NANP)5 peptide to capture anti-(NANP)n antibodies, in an ELISA format. Briefly, (NANP)5 peptide will be coated on ELISA plates, blocked, and incubated with serial dilutions of antisera. An anti-mouse/HRP conjugate, followed by TM Blue substrate will be used to detect bound antibodies.

IFA Testing of Antisera

To test the ability of antisera to bind native sporozoites, immunofluorescence assays (IFA) will be performed on pre-dried slides. Sporozoites (1000/well) will also be coated to ELISA plates for direct antibody binding assays.

Testing Functionality of Universal Malaria T_h Epitope

Although enhanced immunogenicity of HBc-NANP particles containing the malaria universal T_h cell site as compared to particles lacking it will be one indication of "helper" function, direct assays of malaria – T_h cell function will also be performed. Mice will be primed with HBc-$(NANP)_3$-UTC, and the ability of a

UTC peptide to recall T cell proliferation *in vitro* will be determined. Secondly, mice will be primed with the UTC peptide and "challenged" with sub-immunogenic amounts of HBc-(NANP)$_3$-UTC particles *in vivo*. If the UTC-primed T cells are capable of providing T cell help for NANP-specific B cells, enhanced anti-NANP antibody production should occur. This will be determined using the ELISA described above.

Stability Studies

Sterile filtered HBc particles will be incubated at 37 °C. Aliquots will be taken at 0, 1, 3, 7, 14, and 28 days, and analyzed by reducing SDS-PAGE and ELISA. The structure of the 28 day particles will be further analyzed using electron microscopy. Unless there is a clear indication of instability, the most immunogenic of the four particles tested (as determined using non-incubated particles) will be used to immunize mice following a 28 day incubation at 37°C. In the unlikely event that the most immunogenic particle is less stable, or affords a poor yield of purified particles, an alternative particle will be selected for this experiment.

E. HUMAN SUBJECTS

No human subjects or human derived samples will be utilized during phase I.

F. VERTEBRATE ANIMALS

No vertebrate animals will be used at ICC's facility. All animal work will be contracted out to HTI-BioProducts, Ramona, CA, which has NIH approved facilities.

G. CONSULTANTS

Founding Scientists: The founding scientist of ICC include David Milich (Scripps Research Institute), Florian Schodel (Merck), Ben Thornton (ICC), Ashley J. Birkett (ICC), and Darrell Peterson (Virginia Commonwealth University). With the exception of Dr. Schodel, due to his employment at Merck, all ICC founders remain actively involved in the development of the patented HBc platform.

Scientific Advisory Board: Further, ICC has recruited a highly regarded team of scientists for its scientific advisory board (Lindsey Whitton, Ph.D., Scripps Research Institute), James Bittle, Ph.D. (Retired, former President of Pitman-Moore), Fred Brown, Ph.D. (USDA, Plum Island), John Gerin, Ph.D. (Georgetown University), Ruth Nussenzweig, Ph.D. (NYU).

Process Development & Manufacturing: Prima Pharm, San Diego CA.

Preclinical Development: The Company has engaged International Pharmaceutical Services, Inc.("IPS") (Bridgewater, NJ) to oversee the preclinical development of R15K. IPS is a consulting organization for the pharmaceutical and medical device industries worldwide. IPS will be providing expertise to the company in the areas of preclinical development, product development, quality assurance and international regulatory affairs. The principals, Drs. Paul Wray and Jim Davis, have over thirty years experience in pharmaceutical development at Lederle, Wyeth Ayerst, Ortho Pharmaceutical Corporation (Johnson & Johnson), Ortho Biotech and Schering. Additionally, Dr. Wray is an elected Fellow of the Academy of Pharmaceutical Science, where he chairs the Industrial Pharmaceutical Technology Section, and the American Association of Pharmaceutical Scientists.

Clinical Development: The Company has engaged Advanced Biologics, LLC (Lambertville, NJ) to oversee the clinical development of its products. Advanced Biologics is a full service CRO and offers clinical trial and medical management services to the pharmaceutical industry. The principal of Advanced Biologics is Dr. Michael Corrado. Dr. Corrado was formerly Senior Director of Infectious Diseases and later Vice President of Regulatory Affairs for R.W. Johnson Pharmaceutical Research Institute (a Johnson & Johnson company). He currently sits on the ICCAC planning committee as well as the Infectious Disease Society of America (IDSA) FDA Antiinfective Guidelines Committee. Dr. Corrado has extensive experience in designing and implementing clinical strategies, from the preclinical stage through NDA filings. Advanced Biologics currently provides clinical and regulatory support for a number of small

biotechnology companies as well as large pharmaceutical companies (J&J, Merck, American Home Products, and others).

H. CONTRACTUAL ARRANGEMENTS: None

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Checklist

TYPE OF APPLICATION *(Check appropriate box[es].)*

[X] NEW application. *(This application is being submitted to the Public Health Service for the first time.)*

[ ] REVISION of previously-submitted application number _____
(This application replaces a prior unfunded version of a new application.)

[ ] CHANGE of Principal Investigator *(if applicable)*
Name of former Principal Investigator _____

1. ASSURANCES/CERTIFICATIONS

The assurances/certifications set forth below are made and verified by the signature of the OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (small business concern) on the FACE PAGE of the application. Descriptions of individual assurances/certifications are found in application instructions under "Checklist." If unable to certify compliance with any item, provide an explanation and place it after this page.

• Human Subjects; • Vertebrate Animals; • Debarment and Suspension; • Drug-Free Workplace; • Delinquent Federal Debt; • Research Misconduct; • Civil Rights (Form HHS 690); • Handicapped Individuals (Form HHS 690); • Age Discrimination (Form HHS 690).

2. PROGRAM INCOME *(See discussion in application instructions under "Checklist.")*

All applications must indicate *(Yes or No)* whether program income is anticipated during the period for which grant support is requested.

[X] No [ ] Yes *(If "Yes," use the format below to reflect the amount and source(s) of anticipated program income.)*

Budget Period	Anticipated Amount	Source(s)

3. INDIRECT COSTS *(See discussion in application instructions under "Checklist.")*

Insert the rate, if known. If the applicant organization does not have a currently negotiated rate with the Department of Health and Human Services (DHHS) or another Federal agency, it must estimate the amount of indirect costs allocable (applicable) to the proposed Phase I project. That amount should be inserted in the space provided below. The applicant organization should also be prepared to furnish financial documentation to support the estimated amount, if requested by the Public Health Service. An applicant organization may elect to waive indirect costs if it so desires.

[ ] DHHS agreement, dated: _____. _____ % salary and wages or _____ % Total Direct Costs.

[ ] No DHHS agreement, but rate established with _____, dated: _____

[X] Rate negotiation pending with the National Institutes of Health.

[ ] Indirect costs allocable (applicable) to this Phase I project are estimated to be $ _____

[ ] No indirect costs requested.

4. SMOKE-FREE WORKPLACE

Does your organization currently provide a smoke-free workplace and/or promote the non-use of tobacco products or have plans to do so?

[X] Yes [ ] No *(The response to this question has no impact on the review or funding of this application.)*

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